Universidade de Aveiro Departamento de Biologia
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Sofia Maria de Albuquerque Cruzeiro

Isolamento e caracterização de bactérias halófilas de salinas da Ria de Aveiro

Isolation and characterization of halophilic bacteria from salt pans of Ria de Aveiro

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica da Professora Doutora Maria Ângela Sousa Dias Alves Cunha, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro

Dedico este trabalho aos meus pais e irmãos

o júri

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palavras-chave

bactérias halófilas, salinas, biossurfactantes, sideróforos, biocontrolo, quorum-quenching, fosfatase, esterase

resumo

Os microrganismos halofílicos e os metabolitos envolvidos nas suas estratégias adaptativas apresentam uma ampla gama de potenciais aplicações biotecnológicas e, tal como outros ambientes extremos, os habitats hipersalinos são vistos como um reservatório de novos compostos bioativos. O objetivo deste trabalho foi avaliar o potencial biotecnológico de bactérias halofílicas de salinas da Ria de Aveiro em termos de produção de biossurfactantes, características de promoção do crescimento de plantas, efeito de *quorum-quenching* e atividade enzimática extracelular.

No início do outono de 2016, quando a concentração de sal atinge os valores máximos colheu-se água do tanque de cristalização de uma salina ativa da Ria de Aveiro (Marinha de Santiago da Fonte). Utilizando uma abordagem dependente de cultivo, foram isoladas 14 estirpes bacterianas que foram posteriormente identificadas por sequenciação de fragmentos de genes de rRNA 16S como espécies de *Bacillus*, *Halobacillus, Idiomarina* e *Marinobacter*. Para avaliar o potencial biotecnológico destas halófilas, testou-se a produção de biossurfactantes, características de promoção do crescimento de plantas (mobilidade, efeito de biocontrolo e produção de sideróforos), efeito de *quorum-quenching* e atividade enzimática extracelular (fosfatase e esterase). Nenhum dos isolados demonstrou efeitos tensioativos ou de emulsificação significativos que pudessem ser interpretados como indicativos da produção de biossurfactantes. No entanto, todos os isolados exibiram mobilidade, mesmo na presença de 10% de NaCl e produziram sideróforos. Todas as estirpes de *Idiomarina* e *Marinobacter* causaram inibição do crescimento do fungo fitopatogénico *Alternaria*. A inibição de *quorum sensing* também foi detetada em isolados dos 4 géneros, embora com diferenças entre espécies e, em alguns casos, dependendo da salinidade do meio de cultura. A atividade de esterases e da fosfatases foi detetada em todos os isolados e as mais altas taxas de hidrólise dos substratos modelo foram observadas nas espécies de *Halobacillus*.

Embora a produção de biossurfactantes não tenha sido demonstrada, os isolados halofílicos exibem um conjunto de características interessantes em termos de promoção do crescimento de plantas, com potencial aplicação no campo emergente da agricultura salina.

keywords halophilic bacteria, salt pans, biosurfactants, siderophores, biocontrol, quorum-quenching, phosphatase, esterase

abstract

Halophilic microorganisms and the metabolites involved in their adaptive strategies offer a wide variety of potential biotechnological applications and like other extreme environments, hypersaline habitats represent reservoirs of new bioactive compounds.

The main objective of this work was to assess the biotechnological potential of halophilic bacteria from traditional salt pans of Ria de Aveiro in terms of biosurfactant production, plant growth promoting traits, quorum-quenching effect and extracellular enzymatic activity.

Water from an active salt pan of Ria de Aveiro was collected in early autumn when the concentration of salt was at the saturation level. Using a culture-dependent approach, 14 bacterial strains were isolated and identified by sequencing of 16S rRNA gene fragments as species of *Bacillus*, *Halobacillus*, *Idiomarina* and *Marinobacter*. In order to assess the biotechnological potential of these halophiles, they were tested for biosurfactant production, plant growth promoting traits (motility, biocontrol effect and siderophore production), quorum-quenching effect and, activity of extracellular enzymes (phosphatase and esterase).

None of the isolates demonstrated significant tensioactive or emulsification effects that could be interpreted as indicative of biosurfactant production. However, all isolates were motile even in presence of 10% NaCl and produced siderophores. All *Idiomarina* and *Marinobacter* strains caused growth inhibition of the phytopathogenic fungus *Alternaria*. Quorum sensing inhibition was also detected in isolates of the 4 genera although with differences between species and, in some cases, depending on the salinity of the culture medium. Esterase and phosphatase activity was detected in all isolates and maximum hydrolysis rates of the model substrates were the highest in *Halobacillus* species.

Although biosurfactant production could not be demonstrated, the halophilic isolates displayed an interesting set of features in terms of plant-growth promoting traits with potential application on the emerging field of saline agriculture.

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List of abbreviations

ACC - 1-aminocyclopropane-1- carboxylate

AHL - Acylated homoserine lactones

BSF - Biosurfactants

CAS - Chrome azurol S

CTAB - Cethyltrimethylammonium bromide

CFU - Colony forming unit

DNA - Deoxyribonucleic acid

HCN - Hydrogen cyanide

$H_{máx}$ - Maximum hydrolysis rate

MUF - methylumbelliferone

OO - Olive oil

PCR - Polymerase chain reaction

PGPB - Plant growth promoting bacteria

QS - Quorum sensing

QQ -Quorum quenching

rRNA - Ribossmal ribonucleic acid

SA - Saline agar

SB - Saline broth

Introduction

1. Introduction

1.1. Extremophiles

Until the middle of the twentieth century scientists thought that life was only possible under mild conditions, temperate climate temperatures, neutral pH, 1 atm pressure and salinity between freshwater and sea water (1). However, in the last decades it has become clear that life, like microbial, can also occur in extreme conditions. These organisms are called extremophiles (2) and are able to live and reproduce in the most inhospitable environments (3).

Extreme conditions can be defined by temperature (> 45 ° C, <15 ° C), pressure (> 500 atmospheres), salinity (> 1.0 M NaCl), pH (> pH 8.5, <pH 5.0), presence of toxic compounds, organic solvents, heavy metals, exposure to intense radiation and many other factors acting at habitats that may be considered non-receptive to life (2, 4, 5). Most of extremophile organisms cannot live in mild environmental conditions, although there are exceptions that present some flexibility and adaptability (6).

Extremophiles are found in the three domains of life, among Archaea, Bacteria and Eukarya. The former, are now known as the ubiquitous domain. The latter contains interesting examples of extremophiles, such as the unicellular green algae *Dunaliella* that grows in salt-saturated environments, such as the Dead Sea (6, 7).

Thermophiles are extremophiles growing at high temperatures being *Methanopyrus kandleri* the organism known to grow at the highest temperature (122 °C). Organisms that grow at acidic pH values around 3 are named acidophiles (e.g. *Acidithiobacillus* that thrives in acidic ponds or sulfur springs), while those growing optimally at around 12 are called alkaliphiles (e.g. *Bacillus pseudofirmus* present in ecological niches like alkaline soda lakes). There are also organisms that prefer the combination of acidic conditions and high temperatures (thermoacidophiles like *Thermoplasma acidophilum* present in solfataras), while others prefer alkaline conditions and high temperatures, (thermoalkaliphiles like *Anaerobranca gottschalkii).* Organisms that prefer high pressure conditions are called piezophiles (e.g. *Shewanella piezotolerans* isolated from deep-sea

sediments). Those that prefer high salinity conditions are named halophiles like *Salinibacter ruber* that can be found in solar salterns (1, 8–14).

With the advent of the genomic era, the large biodiversity of extreme habitats was revealed. Extremophiles and their metabolites may play a very important role in biotechnology and have several applications namely in agriculture, and in the chemical, biomedical and pharmaceutical industries (4, 14).

The best-known and most successful example of application of an extremophile-derived product in biotechnology is the DNA polymerase isolated from *Thermus aquaticus* from the Yellowstone National Park. This is used for DNA amplification in the polymerase chain reaction (PCR). The discovery of this enzyme opened new perspectives in molecular biology (3, 4, 6, 14).

1.2. Halophiles and their biotechnological applications

In the last years, halophilic bacteria gained attention in the perspective of their biotechnological applications with economic profitability, ecological and phylogenetic characteristics, physiological adaptations to extreme conditions, production of bioactive compounds, and more recently, in the characterization of new enzymes and biochemical mechanisms. Halophilic microorganisms offer a wide variety of potential applications in various fields of biotechnology. The extreme conditions of this type of environment exert force on bacteria selection leading to the expression of adaptive strategies and the synthesis of new metabolites (15, 16).

It is known that proteins produced by extremophiles are more stable in atypical conditions, representing high economic potential in industries such as agriculture, chemistry and pharmaceutics (1, 2). Other compounds from halophilic prokaryotes such as rhodopsins, exopolysaccharides, hydrolases and biosurfactants also have remarkable potentials in the industry (17).

Halophiles are found in all hypersaline environments and are represented in the three domains of life (6). They can be divided into three large groups according to their optimal

growth rates: slightly halophiles (2-5% NaCl), moderately halophiles (5-20% NaCl) and extremely halophiles (20-30% NaCl) (6, 17).

In order to survive high concentrations of salt, these microorganisms must be able to maintain the osmotic balance (2). Osmoregulation in prokaryotes is achieved by two main mechanisms, commonly designated as "salt-in" and "salt-out" strategies. The "salt-in" strategy (17) corresponds to the accumulation of salts, such as potassium chloride (18) within cells at concentrations not lower than the external environment and is used only by some specialized groups of halophiles. In Archaea this model of life is found in the family Halobacteriaceae, whilst in bacteria, for example, can be found in the genus *Salinibacter* (6). Usually halophiles using this strategy exhibit an excess of acidic amino acids (Glu, Asp) over basic amino acids (Lys, Arg), and the amount of hydrophobic amino acids is also relatively low. These proteins are not only soluble and functional with high salt content, but also require molar concentrations of salt for normal activity and stability (6). At low salt concentration, these proteins exhibit remarkable instability (19).

The "salt-out" mechanism, also called the "compatible solute strategy" (17) consists of the exclusion of salts from the cytoplasm and the production or accumulation of organic solutes taken up from the extracellular medium to balance the osmotic pressure of the intracellular medium. The latter is widely used by organisms in the three domains of life and the organic osmolytes may be simple sugars, amino acids or amino acid derivatives, ectoine, glycerol and glycine betaine (17, 18, 20). In Archaea, *Methanohalophilus* species, use glycine betaine and β-amino acids and derivatives, while in Bacteria, glycine betaine is often used as osmotic solute by many different types (18). This mechanism allows a greater degree of flexibility for the organism to grow at a wide range of salt concentrations, therefore, no special adaptation of intracellular proteins is required to function in presence of high concentrations of these solutes (6).

Nowadays, there is an increasing environmental concern and industries are seeking for sustainable solutions and trying to readapt their processes in order to reduce the ecological footprint, since environmental laws have become increasingly intransigent (21). The goal is to move from an industry based on fossil fuels to a sustainable economy based essentially on renewable raw materials. Extremophiles and in particular the

halophiles and their applications have been increasingly pointed as suitable alternatives for a greener chemistry (14). The most direct application of extremophiles in biotechnological processes involves the organisms themselves. Bioleaching, a process in which microbial consortia are used to extract metals such as copper, cobalt, gold and uranium ores, is among the most well established (4). As biocatalysts, extemophiles provide tools for biochemical reactions with high specificity (22). Some strains of halophilic bacteria, such as species of *Marinobacter*, *Idiomarina* and *Halomonas*, are capable of degrading organic pollutants, organic nitrogen compounds and because they are metabolically different and capable of adapting to extreme salinity, can be considered suitable candidates for the bioremediation of hypersaline environments (17).

1.3. Promotion of plant growth

Many microorganisms and their metabolites have a positive effect on plants and contribute to mitigate stress effects and to promote growth. Bacteria that can have this effect are called plant growth promoting bacteria (PGPB) (23). Microorganisms that can grow in, on, or around plant tissues are considered as PGPB (24).

The beneficial effect of PGPB can be direct or indirect. Biofertilization is an example of a direct effect. In this case, microorganisms are used to increase the availability and uptake of mineral nutrients for plants, to control stress through the production of an enzyme capable of decreasing the concentration of stress-induced ethylene, or to stimulate growth of the root system by the production of the hormone auxin. The indirect effects are exerted through competition for nutrients between pathogenic and non-pathogenic microorganisms, through antagonism in which bacteria able to produce antibiotics will inactivate pathogens, and through competition for niches. The biofertilization effect involves the production of bacterial phytohormones, siderophores, or the capacity to fix nitrogen or solubilize phosphate (23, 25–28).

1.3.1. Mitigation of stress

Twenty million hectares of arable land become unusable each year due to salinization of the soil. This is a major threat in the arid and semi-arid regions of the Mediterranean. Thus, the plants thriving in these places begin to suffer stress due to excess salts in the soil, which impairs their growth and development (29).

Plant growth generally exhibits maximum growth periods interspersed with periods of various levels of growth inhibition due to non-lethal stresses (28). PGPB contribute to overcome inhibition of growth by one or more of several different mechanistic strategies (28). PGPB are known to improve plant tolerance to stress such as high salinity, toxic metals, pesticide load and organic pollutants, extremes of temperature, radiation, flooding, drought, wounding, insect grazing and infections with viruses, bacteria, and fungi (28, 29). It has already been shown that *Bacillus aquimaris* is able to alleviated the salt stress in maize (24). *B. pumilus* stimulated the growth of *S. plumbizincicola* in multi-metal contaminated soil (31). *P. putida* was able to reduce hypoxic stress in flooded soils on cucumber plants (32). *Azotobacter* protected cucumber plants against *Cucumber Mosaic Cucumovirus* (33). A bacterial strain of *Bacillus licheniformis* isolated from the rhizosphere of potato showed potential application against phytopathogenic fungi (29).

Plant synthesize ethylene in response to stress, which in turn leads to growth inhibition and premature senescence (28). PGPB expressing the enzyme 1-aminocyclopropane-1- carboxylate (ACC) deaminase, an enzyme that decreases ethylene, can mitigate stress effects and stimulate plant growth in difficult environmental conditions (30). Such bacteria take up the ethylene precursor ACC and convert it into 2-oxobutanoate and NH3 (23, 30). *Pseudomonas mendocina,* which produces ACC deaminase, has been shown to promote the uptake of essential nutrients by salt-stressed lettuce (34).

1.3.2. Nutrient acquisition

Nutrients, water and light are the main factors regulating plant growth (35).

Plants require nutrients such as nitrogen (N), potassium (K), phosphorous (P), magnesium (Mg), calcium (Ca) and sulfur (S), among others, to grow properly (36). Those are known as macronutrients, because they are required by plants in large quantities (37). Micronutrients represent another group of nutrients required in small amount. Iron (Fe), manganese (Mn), copper (Cu), zinc (Zn), nickel (Ni), molybdenum (Mo), boron (B) and chlorine (Cl) are included in the latter group (38). The deficiency in these nutrients may affect plant growth and development, generating, as previously said, poor production yields (39). Only a small portion of the total nutrient content of soil can be utilized by plants (40). The fraction that is biologically available depends on different factors such as season, water content, pH, cation-exchange capacity, redox potential, organic content, microbial activity, and fertilization strategies (40).

In Ria de Aveiro salt pans, soils present high concentrations of salts, especially NaCl. Therefore, plant growth on saline soils is affected mainly by high concentrations of NaCl and impairment of water balance (41). Halophytes are a group that has been able to develop strategies and mechanisms that allow them to thrive in this type of environment (42). In these soils, the bioavailability of some nutrients is limited, since some like phosphorous, occur mainly in their insoluble forms (43). In this way the presence of microorganisms capable of capture them or solubilizing them and make them available to the plant helps to mitigate nutrient limitation (28).

As already mentioned, iron is an important micronutrient for plants, being present in the soil mainly as ferric iron (Fe^{3+}), which cannot be absorbed directly by plants (33, 44). A few microorganisms are able to synthesize and secrete siderophores, a group of small molecular compounds that chelate Fe^{3+} at high specificity to absorb iron in the surrounding environment. The Fe^{3+}-siderophore system is recognized and absorbed by different types of plants, being essential for iron uptake (44). Siderophore production is described in several genera namely P*seudomonas, Azotobacter, Bacillus, Enterobacter, Serrratia, Azospirillum* and *Rhizobium* (45). Siderophores play vital role in promoting plant growth but they can also contribute to prevent plant diseases. They function as a

biocontrol agent by depriving the pathogen from iron (46). For this reason fungal growth is also negatively affected by siderophore-producing PGPB (29).

Although nitrogen is more frequently associated with nutrient limitation in plants, low phosphate levels may also be a limiting factor for growth of salt-stressed plants (34, 47). Phosphorus exists in various forms in the soil, but plants can only absorb their soluble forms, mono and dibasic phosphate. Low availability occurs because phosphorus reacts with calcium, aluminum, iron and manganese to form compounds of low solubility (47, 48). The high use of phosphate and nitrogen fertilizers causes a huge environmental impact. In order to reduce the use of these fertilizers, these bacteria are regarded as biofertilizers representing an alternative to a better use of the natural compounds in the soil (49, 51). Fungi and bacteria are able to solubilize phosphate from various forms of inorganic phosphate, by different biochemical mechanisms. Enzymes, such as nonspecific phosphatases, phytins, phosphoratases, are often involved, but the main mechanism of phosphate solubilization is through the release of organic acids, that through their hydroxyl and carboxyl groups, chelate the cations bound to phosphate and with this are able to release soluble phosphorus in the soil (23, 47, 50). Some examples of phosphate solubilizing bacteria are *Azotobacter, Bacillus, Kushneria, Ralstonia, Rhizobium, Bradyrhizobium, and Thiobacillus.* Some fungi such as *Alternaria, Aspergillus, Fusarium, Penicillium* and *Saccharomyces* also have this capability (43, 51).

1.3.3. Phytohormones

Phytohormones, a diverse group of signaling molecules that are active in very low concentrations, are able to regulate a variety of cellular processes in plants. Their principal roles are related to growth, development and nutrient allocation in plants, and they are also able to increase stress tolerance (52, 53).

Plant hormones are classified as auxins, cytokinins, gibberellins, ethylene, abscisic acid, salicylic acid, brassinosteroids and jasmonates. Among these hormones, auxins are the most multi-functional, abundant and responsible for plant growth and development, not only in regular conditions but also but also under stress conditions (52, 54). Auxins,

gibberellins and cytokinins play an important role in plant adaptation to salt stress (52, 55). Several microorganisms, such as bacteria and fungi in the soil and or associated with plants, synthesize growth hormones identical to those found in plants (49). Some PGPB are capable to produce or modulate phytohormones like auxins, gibberellins and cytokinins (28, 56). For example *Azotobacter spp., Rhizobium spp., Pseudomonas fluorescens* and *Bacillus subtilis* produce cytokinins and auxin can be synthesized by *Pseudomonas putida* (28).

1.3.4. Biocontrol

PGPB can control the growth and activity of phytopathogens viruses, bacteria, fungi and nematodes through different mechanisms (28, 57). These mechanisms include the production of antimicrobial substances such as antibiotics or hydrogen cyanide (HCN), the latter inhibiting the electron transport systems. Other mechanisms involve competition for iron through the release of siderophore that restricts this nutrient to phytopathogens inhibiting their growth, or competition for other nutrients and cell wall-degrading enzymes. Inhibition of quorum sensing may also function as a biocontrol strategy against bacteria (23, 58–60). In mungbean, *Pseudomonas fluorescens* had a significant suppressive effect on root infecting fungi and root knot nematode (58). This bacteria protects plants against the pathogens by producing antibiotics such as 2,4-diacetylphloroglucinol. *Pseudomonas fluorescens* was able to control *Fusarium* wilt on cucumber and showed antagonism against *Alternaria alternata* (60).

The production of lytic enzymes involved in cell wall degradation, such as chitinase, protease and β-1,3-glucanase, are documented in several genera such as *Bacillus, Pseudomonas, Stenotrophomonas, Acinetobacter, Enterobacter*, and *Serratia* (59).

1.4. Production of biosurfactants

Surfactants (Surface Active Agents) are molecules composed of two functional groups, the hydrophobic group (oil-soluble) or non-polar group and hydrophilic (water-soluble) or

polar group (61, 62) that are able to reduce surface and interfacial tension. Surfactants are widely used in almost all sectors of industry for emulsification, foaming, detergency, wetting, dispersing and solubilization (62, 63). Surface active agents are mostly obtained from petroleum through chemical processes (63, 64). However, with the growing environmental concern, environmental friendly alternatives such as biosurfactants (BSF) which are surfactants produced by microorganisms, have been sought (65).

BSF are produced usually as secondary metabolites by bacteria, yeasts and fungi from various substrates, such as sugars, glycerol, oils, hydrocarbons and agricultural wastes (66, 67). Like chemical surfactants, BSF are amphiphilic molecules capable of reducing surface tension and interfacial tension (68). The hydrophobic moiety of the molecule consists of a long chain of fatty acids, hydroxyl fatty acids or a-alkyl-b-hydroxyl fatty acids, while the hydrophilic moiety may be composed of carbohydrate, amino acid, cyclic peptide, phosphate, carboxylic acid or alcohol (67, 69).

BSF are used by microorganisms to solubilize hydrophobic compounds from the medium in order to be used as substrates and have important physiological roles in cell to cell communication and swarming motility (70). The molecules can be released to the extracellular environment maintain a physical association with the cell membrane (70).

Organisms capable of producing biosurfactants have already been isolated from a wide variety of habitats including sea water, marine sediments, fresh water, groundwater, soil, sediment, sludge and different extreme environments (hypersaline environments, oil reservoirs) (68, 70). Typically, BSF from microbial origin are classified as low molecular weight biosurfactants such as glycolipids, lipopeptides and high molecular weight biosurfactants such as polysaccharides, lipopolysaccharides, proteins or lipoproteins (15, 70).

BSF represent an advantage over chemical surfactants in terms of environmental compatibility, low toxicity, biodegradability, bioavailability, activity under various conditions, diversity of molecular structures, simplicity of preparation, ability to be modified by genetic engineering and the ability to increase the bioavailability of few soluble organic compounds (65, 66, 71). Some the industrial applications are related with agriculture, cosmetics, pharmaceutical products, domestic and industrial detergents,

leather, paper and textile production as well as metallurgy and mining (15, 65, 66). These compounds have promising environmental applications namely in oil bioremediation of soils and waters and enhanced oil recovery (EOR). They have also interesting biological effects acting as antibiotics, antiviral, antifungal and antifouling agents controlling biofilm formation (15, 16, 67, 71, 72).

1.4.1. Biosurfactant-producing halophiles

Biosurfactant producers have been identified in three domains of life (73). In Archaea, it has been demonstrated in a *Natrialba sp.* isolated from a salt lake in Algeria (16). *Kocuria marina* isolated from solar salt works in India (68), and also in *Marinobacter, Bacillus* and *Nocardiopsis* (67, 68, 70) are exaples of BSF production in the Bacteria domain. In Eukaryota, BSF production was reported in *Fusarium* sp. isolated from oil contaminated soil samples (74). The search for new biosurfactants in halophilic microorganisms is a promising research field, since these biosurfactants have particular properties that make them more stable in extreme physical or chemical conditions (16, 68).

1.4.2. Applications of biosurfactants from halophilic microorganisms

BSF from halophilic microorganisms can find applications in industrial processes involving high concentrations of salt because in these conditions they are more stable than other types of surfactants (67). Table 1 presents some applications of biosurfactants produced by different halophilic bacteria.

Bioremediation consists in the biological decomposition of hydrocarbons, performed by microorganisms capable of using these molecules as a carbon source to obtain energy, degrading them into water, mineral salts, carbon dioxide and gases (75). This method is one of the most environmentally-friendly, cost effective and sustainable strategy to clean environments polluted with organic compounds (76). BSF produced by halophytes increase the bioavailability of pollutants to plants and microorganisms in saline ecosystems, reducing surface tension and increasing the solubility of these

compounds, thus facilitating their removal (25, 77). Biosurfactant-producing halophiles may play an important role in the remediation of saline environments contaminated with petroleum (78). A study reported that *Bacillus subtilis* BS2, a halophilic biosurfactant-producing bacteria, is capable of enhancing bioremediation in oil contaminated sites, including in marine environments (79).

Table 1. Summary of different types of BSF produced by different halophilic bacteria and their applications.

Organism	**Application**	**BSF type**	**Reference**
Bacillus sp. BS3	Pharmaceutical: Antibacterial and antifungal activity	Lipopeptide	(15)
Bacillus tequilensis CH	Pharmaceutical: Inhibition of biofilm formation	Lipopeptide	(80)
Nesterenkonia sp.	Food industry: antioxidant activity and antibiofilm	Lipopeptide	(81)
Natrialba sp. C21	Bioremediation: Mineralization of aliphatic and aromatic hydrocarbons in contaminated systems	Non determined	(82)

Some BSF have antimicrobial properties that act in the biological control of fungi can also improve nutrient uptake by increasing the bioavailability of hydrophobic molecules which may serve as nutrients (23, 83, 84). Surfactin has antimicrobial activity against bacteria and fungi by accumulating on the surface of the microbial cell and achieving a concentration that is high enough to work like a detergent permeating the membrane and leading to its disintegration (85). A BSF produced by *Pseudomonas*

aeruginosa showed to be an efficient antifungal agent to prevent wilt disease in tomato plants (85).

1.5. Production of enzymes

Halophiles thrive in environments with high salt concentrations and therefore they express particular biochemical adaptations. That is the case of enzymes that by containing high number of negatively charged amino acid residues to avoid precipitation (2). Thus the solubility of these enzymes is poor, this characteristic was used for biotechnological application (2). The enzymes produced by halophiles are stable not only at high salt concentrations but also at high temperatures and in the presence of organic solvents (86). These characteristics make them an attractive alternative for food processing and environmental bioremediation (78, 85).

Hydrolases like lipases, esterases, proteases and nucleases derived from halophiles, have been reported to have potential use in industry (87). Lipases and carboxilesterases are extensively produced and well distributed in nature and are the most representative classes. Lipases and esterases are lipolytic enzymes and the difference between them lies on the substrates that they are able to recognize and on the interface where these enzymes act. The former hydrolyze long-chain acylglycerols and catalyze the reactions at the oil–water interface, whilst the latter hydrolyse ester substrates with short-chain fatty esters and catalyze reactions of water-soluble substrates (14, 88). *Halomonas sp*. isolated from Spanish and Turkish saltworks was capable of producing both lipase and esterase (86).

Extracellular phosphatases are important for marine bacteria because they play an important role in the recycling of organic phosphate and avoid their limitation in the extracellular medium. These enzymes have the ability for hydrolyzing a variety of organic phosphorus compounds into orthophosphate and alcohol (89). In addition, phosphatases also play an important role in the degradation of petroleum (90).

Isolates from the genera *Bacillus*, *Halobacillus, Idiomarina* isolated from fermented fish and salted fish are able to produce phosphatase (91).

1.6. Other applications

Halophilic bacteria can also produce other types of biologically active compounds like antimicrobials and anti-oxidants (92, 93). *Alteromonas sp.* produces the antibiotic 2-n-pentyl-4-quinolinol, which has the ability of influencing bacterial community and their metabolic and physiological parameters (94). *Kocuria sp*. strain QWT-12 is a carotenoid-producing organism, which is a pigment with antioxidant and antitumor activity (95).

Quorum sensing (QS) is a mechanism through which bacteria can control specific mechanisms in response to population density (96) and regulate metabolism as response to biotic and abiotic changes (56). In pathogenic microorganisms, quorum sensing allows the synchronization and expression of the virulence genes. This occurs through the production, release and detection of signaling molecules that act as autoinducers (96, 97). Quorum quenching (QQ) is the mechanism that interferes with the successful chemical communication process (quorum sensing) and it has been regarded the control of infections in a context of resistance to antibiotics, because it does not interfere with microbial growth, but rather inhibits the expression of virulence (97). According to the literature, QQ can occur mainly by blockage of the synthesis of auto-inducers, degradation of auto-inducers or interference with the signal receptors (98). Three different groups of enzymes are involved in AHL-signal molecules degradation, the AHL lactonases, the AHL acylases, and the AHL oxidases/oxidoreductases (99) .

This interference in bacterial communication can be considered a biocontrol mechanism (23) and the production of quorum quenchers represents a natural, widespread, antimicrobial strategy (96). Inoculation of plants with bacteria capable of inhibiting quorum sensing has already been shown to be effective against pathogenic bacteria and against saline stress (100). *Halobacillus salinus*, showed inhibition of AHL in

biosensor *Chromobacterium violaceum* through the production of N (2'-phenylethyl)-Isobutyramide 3-methyl-N (2'-phenylethyl)-butyramide compound (101). *Hyphomonas sp.*, produces acylase and lactonase capable of degrading the AHL molecules (102). *R. erythropolis* degrades AHL through oxidoreductase and acylase activities (103).

2. Objectives

The aim of this work was to assess the biotechnological potential of halophilic bacteria from salt pans of Ria de Aveiro. For that, a culture dependent approach was used to obtain isolates that were tested for biosurfactant production, general plant growth promoting traits, extracellular enzymatic activity and quorum-quenching effect.

Methods

3. Methods

3.1. Study area and sample location characterization

The Santiago da Fonte salt pans (Marinha de Santiago da Fonte) represent a 5 ha area of marsh classified as a special protection zone as a reproduction and feeding site for a large number of bird species. This area was acquired by Aveiro University in 1993 and it is one of the seven salt pans still operating in the artisanal extraction of salt in Ria de Aveiro. The number of active salt pans in the 80s was around 270.

All samples were collected in 21st of September 2016 at a sampling site with the coordinates 40.628676 N, 8.660874 W (Figure 1).

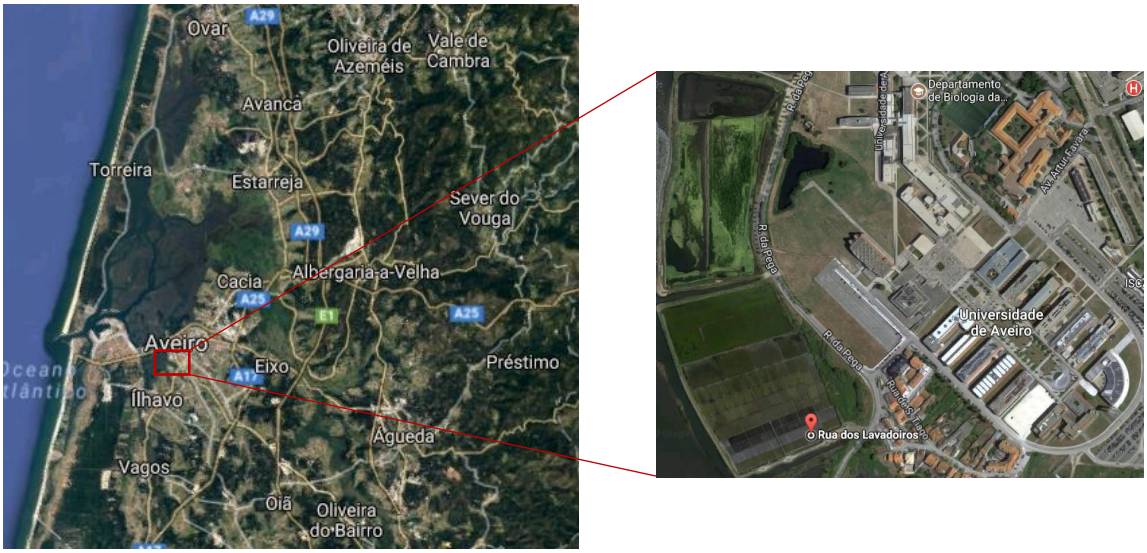

Figure 1. Santiago da Fonte salt pans. The sampling site is marked in red.

3.2. Sample collection, isolation and purification of halophilic bacteria

A sample of 1 L of water was collected with a 1 L glass vial. Salinity was determined with a refractometer (TMC V^2 Refractometer) and confirmed with a salinometer (Cond 3110 set 1, WTW).

The isolation of bacteria was conducted by preparing serial dilutions (10^{-1}, 10^{-2}) in Ringer solution (Merck, pH = 7.0 ± 0.2) and pour-plating 100 µL-aliquots of each dilution in Saline

Agar (SA100). This medium was prepared from Marine Broth (Difco) by adding 1.5 % agar and water from the salt pans, previously sterilized, to provide micronutrients and achieve a final salinity of 100‰. The pH was adjusted to 7.6±0.2. In subsequent tests, Saline Agar or Saline Broth with salinities 20‰ and 100‰ were prepared by adjusting the salinity with NaCl (92).

The plates were incubated for 96 hours at 37 °C and isolated colonies were selected based on morphology and colour for purification on SA, using the standard streaking technique. New cultures were incubated at 37 °C for 96 hours. The purity of the cultures was confirmed by optical microscopy after gram staining. Finally, liquid cultures were prepared in Saline Broth with salinity 100 ‰ (SB100) and stored at -80ºC with 20% glycerol (AppliChem) as a crio-protector.

3.3. Molecular identification of isolates

3.3.1. DNA Extraction

For the DNA extraction, an adapted method of Gomes *et al.* (2004) (104) was applied. A volume of 1.5 mL of liquid culture in Saline Broth of each isolate was centrifuged at 16,000 x g (Heraeus Pico 17 centrifuge, Thermo scientific) for 5 minutes. The pellet was resuspended in 800 µL of ethanol 96% and then transferred to Fast Prep tubes containing 500 mg of glass beads. The tubes were agitated for 2 consecutive periods of 30 seconds in the Thermo Savant FastPrep 120 Cell Disrupter System at 5.5 m/s and later centrifuged at 16,000 x g for 5 minutes. The supernatants were discarded and 1mL of extraction buffer (1% CTAB- cethyltrimethylammonium bromide, 2% sodium dodecyl sulfate, 1.5 M NaCl, 10 mM sodium phosphate buffer pH 7.0, 10 mM Tris-HCl pH 7.0, 1 mM EDTA pH 8.0) was added. The mixture was gently mixed and incubated for 15 minutes at 65 °C. The tubes were centrifuged again for 5 minutes at 16,000 x g and the supernatants transferred to new 2 mL microtubes containing 1 mL of phenol-chloroform-isoamyl alcool (25:24:1). The tubes were carefully mixed and again centrifuged at 16,000 x g for 5 min. The resulting aqueous phase was then transferred to a new microtube

containing 0.6% isopropanol (vol/vol) and incubated for 30 min at room temperature. A final centrifugation of 20 minutes at 16.000 x g was performed, the supernatants were discarded and the tubes were incubated at 55 °C for 20 minutes to allow the reminiscent liquid to evaporate. Finally, the pellet was resuspended in 80 µL of TE buffer (10 M Tris-HCl pH 7.4) containing 1 mM EDTA.Na_2 (pH 8.0) and the tubes stored at -20ºC.

3.3.2. BOX-PCR

Molecular typing of the isolated strains was conducted by BOX-PCR using a protocol based in the method described by Martin *et al.* (1992) (105). The final volume of 25 µL of the mixture was composed of 8.75 µL dH_2O, 1.25 µL DMSO, 1.50 µL primer BOX_A1R (5'-CTA CGG CAA GGC GAC GCT GAC G-3') and 12.50 µL of Master mix (Thermo Fischer Scientific). The cycling conditions used included a denaturation step of 7 min at 94 °C, followed by 35 thermal cycles of 1 min at 94 °C, 2 min at 53 °C, and 8 min at 65 °C, and finally an extension step at 65 °C for 16 min. The PCR products were stored at -20 °C.

The success of the BOX-PCR was confirmed by running an electrophoresis in agarose (Gentaur) gel (1.5%) with 5µL of RedSafe™ as a staining agent of the DNA, for 120 minutes at 80 volts in 120 mL of 1xTAE buffer (0.04 M Tris-Acetate, 0.001 M EDTA Fluka; pH 8.0). Bands were visualized in a UV transiluminator (Benchtop UV). The profiles obtained were analysed with GelCompar 4.0 software (Applied Maths, Belgium).

3.3.3. PCR-amplification of 16S rRNA gene fragments

PCR-amplification was conducted with the primers 1494R (5'- TAC GGC TAC CTT GTT ACG AC -3') and 27F (5'- AGA GTT TGA TCC TGG CTC AG -3'). The final volume of 25 µL of the mixture was composed of 1 µL of sample, 12.5 µL DreamTaq™ PCR Master Mix, 0.25 µL of primer 1494R, 0.25 µL of primer 27F, 1 µL BSA (Bovine Serum Albumin, 2 mg/mL, Sigma) and 10 µL dH_2O. The cycling conditions used included 5 min of denaturation at 94 °C followed by 35 thermal cycles of 45s at 94 °C, 45s at 56 °C and 90s at 72 °C, and a final extension step at 72 °C for 10 min. The success of the amplification of the 16S rRNA gene

fragments was confirmed by running an electrophoresis, in agarose gel (1.5%) with 5 µL of RedSafe™ as DNA staining agent the, for 30 minutes at 80 volts in 120 mL of 1xTAE buffer. Bands were visualized in a UV transilluminator (Benchtop UV). The amplicons were sequenced by StabVida. The obtained sequences were matched to the sequences available in the GenBank database using BLAST (Basic Local Alignment Search Tool; http://www.ncbi.nlm.nih.gov) to determine their closest relative.

3.4. Biosurfactant production

The production of biosurfactants was tested through two of the most used qualitative methods (72). Cethyltrimethylammonium bromide (CTAB 0.2 mM) was used as positive control and the liquid culture medium was used as negative control.

3.4.1. Drop collapse assay

This method is adapted from Bodour and Miller-Maier (1998) (106) and it is based on the principle that a drop of water collapses (and the diameter increases) in presence of a surfactant-containing extract. Isolated strains were cultivated at 37 °C for 48 h in four different media, SB20 (salinity 20 ‰), SB100 (salinity 100 ‰), SB20-OO (salinity 20 ‰ + 2% of olive oil) and SB100-OO (salinity 100 ‰ + 2% of olive oil). After the incubation, 1.5 mL-aliquots of cultures were centrifuged for 5 minutes at 16,000 x g (Heraeus Pico 17 centrifuge, Thermo scientific). The supernatants were used for the assay. A 5 µL droplet of distilled water was placed on a plastic petri dish, previously cleaned with distilled water and ethanol (97%). One-microliter aliquots of sample or controls were added at the centre of the water droplet. The droplet stabilized for 1 minute and was later examined using a magnifying glass (Olympus cover-015) for determination of the diameter. Measurement was performed after calibration of the 16X objective, using an ocular micrometer and a micrometric ruler. Two independent trials were done, with 5 replicates each.

3.4.2. Emulsification index

Biosurfactant production was also assessed by estimating the emulsification index, according to the method described by Cooper and Goldenberg (1987) (107). Isolates were cultivated at 37 °C for 48 h in the 4 different media previously described (SB20, SB100, SB20-OO and SB100-OO). Two mL of liquid paraffin (Merck) and 2 mL of each culture were mixed in a tube, centrifuged for 2 minutes in the vortex (Labinco) and allowed to stand for 24 h. Biosurfactant production was detected from the formation of a foam layer. The thickness of the foam layer was measured with a millimetric scale. The value was divided by the total height of liquid in the tube and multiplied by 100 to obtain an emulsification index expressed as a percentage. Ringer solution, distilled water and each of the different media used for the cultivation of the isolates were used as negative controls and a 0.2 mM CTAB solution was used as positive control. Three independent trials were performed.

3.5. Plant growth promoting traits

To assess the plant growth promoter potential isolates were tested for isolates were tested for motility, biocontrol effect and siderophore production.

3.5.1. Motility

Cell motility was observed with an optical microscope with a total magnification of 1500X (Leitz Laborlux K) in fresh mounts obtained by adding 5 µL of fresh culture and 5 µL of Ringer solution to a microscope slide.

3.5.2. Biocontrol effect

Bacterial isolates were tested for potential antifungal properties against the phytopathogenic fungus *Alternaria sp.* adapting the method described by Sgroy *et al.*

(2009) (108). The centre of a plate of medium SA20 was inoculated with a 6 mm-diameter disk of mycelium of a fresh culture of *Alternaria*. Two blank antibiogram disks were placed on the edge of the plate, equidistantly from de centre. One contained 25 µL of fresh culture of the test bacterial strain (T) and the other contained 25 µL of sterilized dH_2O as negative control (C). The plates were incubated for 13 days at room temperature (approx. 25 °C). Mycelium growth inhibition (I) was calculated as I = [(RC-RT)/RC] × 100, where I = mycelia growth inhibition (%), RC = radius of fungal mycelium growing towards disk C and RT = radius of fungal mycelium growing towards disk T. Three independent trials were performed. Fluconazole was used as a positive control.

3.5.3. Siderophore production

The method for evaluating siderophore production was adapted from Pérez-Miranda *et al.* (2007) (109). An aliquot of a fresh liquid culture of each isolate was streak-plated in SA20. The cultures were incubated for 48h at 37ºC. A *Pseudomonas aeruginosa* ATCC® 27853 culture was used as positive control and non-inoculated SA20 plates were used as negative controls. Siderophore production was detected by adding 5mL of chrome azurol S (CAS) medium [CAS 60.5 mg/L, CTAB 72.9 mg/L, piperazine-1,4-bis (2-ethanesulfonic acid) (PIPES) 30.24 g/L, 1 mM $FeCl_3.6H_2O$ in 10 mM HCl 10mL/L and 0.9% (w/v) agarose] over the plates, which were then further incubated for 24h at 37ºC. A change in the color of the overlaying CAS medium from blue to orange/yellow or the formation of yellow halos surrounding the culture was interpreted as a positive result.
Three replicates were performed.

3.6. Quorum quenching

This method to evaluate the inhibition of quorum sensing (quorum quenching) was adapted from McLean *et al.* (2004) (96). The indicator strain, *Chromobacterium violaceum* was grown in Luria Broth medium (LB, Liofilchem, pH = 7.0 ± 0.2) at 37 ºC for 24 h. After growth, 100 µL of the culture were spread-plated on the surface of Tryptic Soy Agar (TSA,

Liofilchem, pH = 7.2 ± 0.2) plates. Fresh cultures of bacterial isolates, grown at media SB20 and SB100, were centrifuged at 16,000 x g for 5 min (Heraeus Pico 17 centrifuge, Themo scientific) and blank disks were soaked in the supernatant and placed on top of TSA medium. A disk soaked in cinnamaldehyde (Merck) was used as positive control and another disk soaked in the indicator strain (*C. violaceum*) was used as negative control. The formation of depigmented halos surrounding the inoculated disks was interpreted as quorum sensing inhibition.

3.7. Activity of extracellular hydrolytic enzymes

To evaluate the extracellular enzymatic activity a method adapted from Hope (1983) (110), based on the hydrolysis of fluorogenic model substrates, was used.

3.7.1. Determination of the saturation concentration

Aliquots of different MUF (fluorogenic methylumbelliferone) -substrates (MUF – phosphate [10 mM] and MUF – stearate [2 mM]) solutions (2, 4, 6, 8, 12 µL) were transferred to 10 replicate wells in 96 wells plates. A 1:1000 dilution of a fresh liquid culture of *Bacillus licheniformis* used as reference strain, was prepared in Ringer solution with 20 g/L NaCl and 200 µL aliquots were added to the wells containing the substrate solution. Finally, 20 µL of an alkaline buffer (1.384 mL ammonia, 0.375 g glycine, distilled water up to 1 L, pH 10.5) was added to each series of 5 wells immediately before measuring the fluorescence at initial time (t=0h). The plates were incubated for 37 °C (t=2h for MUF-phosphate and t=3h for MUF-stearate) and the buffer solution was added to the series of 5 wells immediately before measuring the final fluorescence. Fluorescence was determined in a spectrophotometer (Cary Eclipse Fluorescence Spectrophotometer, Agilent Technologies) at wavelengths of 365 nm (excitation) and 460 nm (emission). A mean and standard deviation of the initial and final fluorescence was calculated for each concentration of each substrate and the difference between the final

and the initial value was plotted against the substrate concentration to infer on the saturating concentration to be used in further assays.

3.7.2. MUF calibration curve

Aliquots of a 0 µL, 2 µL, 4 µL, 6 µL and 8 µL stock solution of 10 mM MUF (methylumbeliferone stock solution) were transfered to 4 replicate wells of 96-well plates. To each well, 200 µL of a 1:1000 dilution of a fresh liquid culture of *Bacillus licheniformis* prepared in Ringer solution with 20 g/L NaCl was added. Twenty microliters of alkaline buffer were added to each well and the fluorescence intensity was measured. The average intensity for each series of replicate well was calculated and plotted against the concentration of MUF to obtain a calibration curve.

3.7.3. Maximum hydrolysis rate

The maximum hydrolysis rate ($H_{máx}$) of the two MUF substrates mentioned above was calculated using the saturation concentrations determined in the preliminary assay, 0.27 mM for MUF-phosphate and 0.05 mM for MUF-stearate and an incubation time of 2h for MUF-phosphate and 3h for MUF-stearate. A mixture of 6 µL from each MUF substrate (corresponding to a saturation concentration of 270 µM for MUF-phosphate and 50 µM for MUF-stearate), and 200 µL of a 1:1000 dilution of a liquid fresh culture of each bacterial strain, prepared in Ringer solution with 20 g/L NaCl, was transferred to 10 replicate wells. Finally, 20 µL of alkaline buffer was added to a series of 5 replicate wells and the initial fluorescence was read in these wells (t=0h). After incubation, the alkaline buffer was added to the remaining 5 wells and the final fluorescence was determined. The average initial and final fluorescence was calculated and the difference (final-initial) was converted in concentration units using the calibration curve. The $H_{máx}$ was calculated by dividing the concentration value by incubation time. The specific $H_{máx}$ was calculated by dividing the $H_{máx}$ by the concentration of viable cells expressed as colony forming unit (CFU) per volume unit. The concentration of viable cells was determined by serially

diluting the bacterial suspension in Ringer solution with 20 g/L NaCl, pour-plating the most convenient dilutions and determining the average CFU concentration after 24h incubation at 37 °C.

Results and discussion

4. Results and discussion

4.1 Isolation and identification of halophilic bacteria

Taking into account the morphology, color and size of the colonies, a set of 14 isolates was obtained. After the DNA extraction of all the isolates, the molecular typing was carried out by BOX-PCR, to investigate similarities among them, as an attempt to reduce the number of isolates for subsequent sequencing. Only two isolates (#1 and #7) presented similarity higher than 95% (Figure 2) and therefore 13 isolates were sent for 16S rRNA gene sequencing in StabVida.

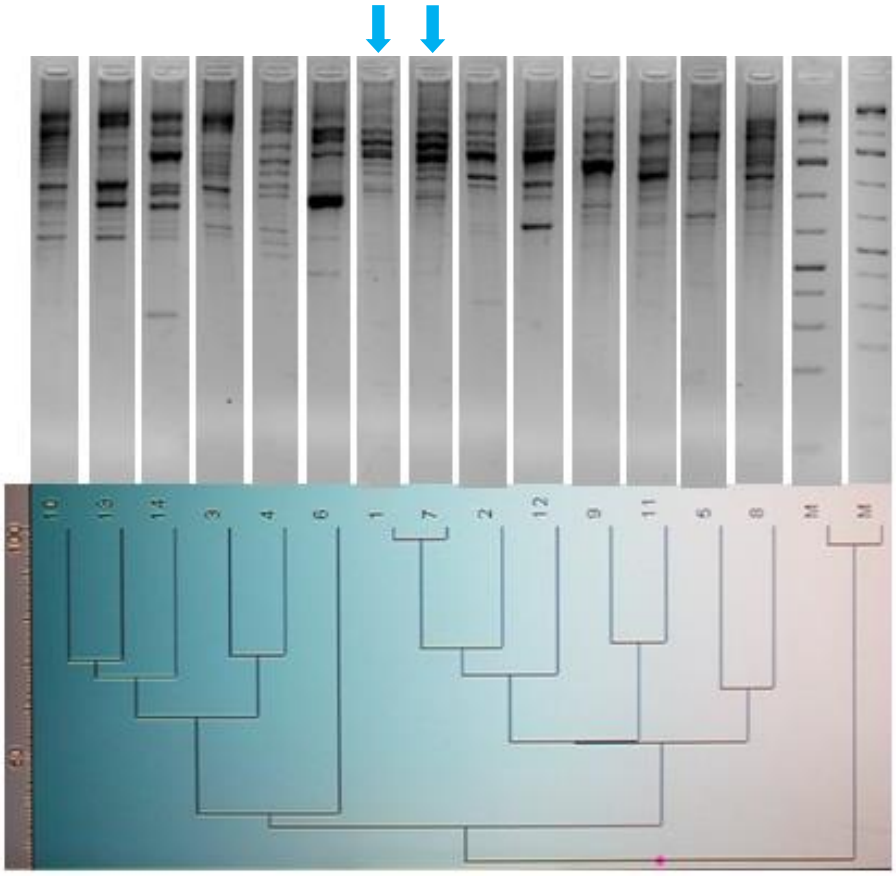

Figure 2. Dendrogram with results of electrophoresis of PCR products with primer BOX_A1R. Each number corresponds to one isolate. M correspond to the markers. The blue arrows represent the two isolates with the highest similarity.

The sequences obtained were compared to the sequences in the Genbank database using BLAST (Basic Local Alignment Search Tool) software. Isolates were assigned to genera *Bacillus, Idiomarina, Halobacillus and Marinobacter* with similarity greater than 95% to sequences deposited in the Genbank (Table 2).

The fact that the isolate #2 exhibits a 95% similarity with the sequences in the database may mean that we are dealing with a new species.

Table 2. Identification of isolates and corresponding percentages of similarity

Isolate	Identified as	% similarity	Accession number
1	*Bacillus licheniformis*	99%	MG575726
2	*Idiomarina sp.*	95%	MG575727
3	*Idiomarina seosinensis*	100%	MG575728
4	*Halobacillus alkaliphilus*	99%	MG575729
5	*Halobacillus locisalis*	100%	MG575730
6	*Idiomarina seosinensis*	99%	MG575731
7	*Bacillus licheniformis*	99%	MG575732
8	*Marinobacter salsuginis*	99%	MG575733
9	*Marinobacter salsuginis*	99%	MG575734
10	*Idiomarina zobellii*	99%	MG575735
11	*Marinobacter adhaerens*	100%	MG575736
12	*Idiomarina seosinensis*	100%	MG575737
13	*Idiomarina seosinensis*	99%	MG575738
14	*Idiomarina zobellii*	100%	MG575739

The results are in line with previous surveys where these bacterial genera have been reported to be present in hypersaline environments. *Idiomarina seosinensis* was found in a hypersaline water sample from a solar saltern in Korea (111), *Halobacillus locisalis* in marine solar saltern located in Baekryung Island of the Yellow Sea in Korea (112), *Halobacillus alkaliphilus* in the Fuente de Piedra salt lake, southern Spain (113).

Marinobacter salsuginis were found in the Red Sea (114), *Marinobacter adhaerens* in aggregates taken from surface waters of the German Wadden Sea (115), and *Bacillus licheniformis* and *Idiomarina zobellii* were isolated from Pacific Ocean water samples from (115, 116).

Phylogenetic analysis based on 16S rRNA gene sequences indicate that the isolated bacteria belonged to two major taxa: Gammaproteobacteria (71%, including *Idiomarina* [50%] and *Marinobacte*r [21.4%]) and Firmicutes (29%, including *Bacillus* [14.3%] and *Halobacillus* [14.3%]). The isolation of a significant proportion of Gammaproteobacteria is consistent with the previously reported abundance of this bacterial class in hypersaline environments (17). *Halobacillus* (118), *Marinobacter* (119), *Idiomarina* (120) and *Bacillus licheniformis* (119) are often found in contaminated environments.

4.2 Biosurfactant production

Biosurfactants produced by bacteria can play a variety of roles such as motility, antagonism, access to hydrophobic substrates, biofilm development and pathogenesis (121). The surface tension reduction and emulsion forming and stabilizing capacity are the most important surface-active properties evaluated in screening for microorganisms with potential industrial application (122). Biosurfactant production in bacterial cultures can be indirectly demonstrated by assessing the effect of cell-free extracts on the reduction on surface tension (tensioactive effect) or the formation of foam in mixtures with hydrophobic compounds (emulsification effect).

The drop collapse assay was performed as qualitative method based on the variation of surface tension (65). In this method, if the culture medium added to a water droplet contains biosurfactant, the drop will collapse and consequently its diameter will increase (66). The average droplet diameters corresponding to the results obtained in samples and controls are represented in figure 1, Appendix A. As expected, the diameter of the drop in the positive control (4.92 mm – 5.25 mm) was significantly larger than the average diameter corresponding to the negative control (non-inoculated culture medium). The drop diameter in the negative controls varied between 2.92 mm and 3.21

mm and in the tests the drop diameters varied from 2.83 mm to 3.82 mm, which is not significantly different from the negative controls (ANOVA, $p>0.05$). Therefore, it was not possible to demonstrate a significant tensioactive effect associated with any of the isolates cultivated either with a moderate (20‰) or a high (100‰) salinity. Olive oil is often used as a substrate for the cultivation of biosurfactant producing bacteria in order to enhance the production of tensioactive substances (67). However, in this work, the addition of 2% olive oil to the cultivation medium did not affect tensioactive properties of cell-free extracts.

The emulsification index was also determined as a quantitative measure of the effect of biosurfactants released to the culture medium (65). The emulsification index establishes the ratio between the height of the foam layer in the test tube and the total height of the liquid (mixture) and it is expressed as given as a percentage. A CTAB solution (0.2 mM) was used as positive control and non-inoculated culture medium was inlcuded as negative control. The results are represented in Figure 2, Appendix A.

An emulsification index > 50% is indicative of a good emulsification capacity (123). As expected, the emulsification index was higher than 50% in the positive control but lower than this (0.0% - 27.7%) in the negative control and in all the tests. Although significant tensioactive or emulsification effects were not demonstrated for any of the isolates under study, biosurfactant production is reported in literature for all the corresponding genera or species. *Marinobacter* isolated from marine sediment from Mediterranean harbors, produces a phosphopeptide detected by the drop collapse assay, emulsification activity and interfacial surface tension (124). *Bacillus licheniformis* isolated from soil samples from Bohai Sea produces a lipopeptide identified by LC-ESI-MS/MS (liquid chromatography-electrospray ionization-tandem mass spectrometry), this biosurfactant showed good resistance to pH, high temperature and UV radiation (125). *Halobacillus* isolated from oil-contaminated mangrove sediments in Red Sea coast, also produces a lipopeptide, tested through haemolytic activity, oil displacement test, surface and interfacial measurements (118). The first report of biosurfactant production by *Idiomarina* was in 2013, in a strain isolated from adult specimens of polychaete annelids from Lake Faro (Italy) and detected by surface tension measurement (120).

The two methods tested analyze different properties of the biosurfactants, where the drop collapse assay gives information about tensioactive activity and the emulsification index is a screening test for measuring the emulsification capacity. Although the methods give different information, biosurfactants often have both effects and therefore this information is complementary.

4.3 Plant growth promoting traits

Some physiological and biochemical features of the isolates were analyzed in the perspective of the capacity of these halophilic bacteria to establish successful and mutually beneficial relations with plants that eventually contribute to the mitigation of saline stress in plants and promote their growth.

The motility of the isolates was assessed by the observation of fresh mounts under the optical microscope and the results are summarized in table 3. All isolates were motile and in general, the salinity of the medium did not appear to affect motility. However, two isolates showed greater motility in the SB100 medium (#8 *Marinobacter salsuginis* and #13 *Idiomarina seosinensis*). The fact that the motility of these two isolates was higher in the medium with higher salinity is in agreement with the literature. It has been demonstrated that in some halophilic bacteria, the concentration of Cl^- influences the development of flagellum, and it has been proposed that chloride can act as an intracellular signal for the transcription of genes responsible for the flagellum, or may be involved in the export and assembly of subunits or in the stability of the flagellum (126). *Idiomarina* and *Marinobacter* isolates were the most motile which agrees with literature reports of motility by means of a single polar flagellum in these genera (110, 113, 115).

Table 3. Classification of isolates according to motility in media SB20 and SB100

Samples	SB20	SB100
1 *Bacillus licheniformis*	++	++
2 *Idiomarina sp.*	+	+
3 *Idiomarina seosinensis*	+	+
4 *Halobacillus alkaliphilus*	-	-
5 *Halobacillus locisalis*	+	+
6 *Idiomarin*a *seosinensis*	++	++
7 *Bacillus licheniformis*	+	+
8 *Marinobacter salsuginis*	+	++
9 *Marinobacter salsuginis*	+	+
10 *Idiomarina zobellii*	++	++
11 *Marinobacter adhaerens*	++	++
12 *Idiomarina seosinensis*	++	++
13 *Idiomarina seosinensis*	+	++
14 *Idiomarina zobellii*	+	+

++ very motile + motile - poorly motile

Bacterial motility is an important feature of plant growth promotion because it is related to capacity to colonize plants roots, to form biofilms and to chemotactic behaviour in relation to nutrient gradients which are determinant for the success of plant-bacteria relations in the rhizosphere (23, 120, 126). Also, the production of biosurfactants by bacteria influences their motility. Biosurfactant-producing bacteria are usually more motile because the biosurfactant reduces the shearing force of the cells on the fluid or surface and allows gliding motility in addition to facilitating the activity of flagella (70, 83, 127).

The biocontrol effect mediated by the release of antimicrobial or allelopathic substances is an important aspect of the mechanisms of plant-growth promotion, namely by preventing the establishment and development of phytopathogenic fungi (30). Several mechanisms may be used by bacteria for inhibition of phytopathogenic fungi including competition for nutrients and hydrolytic enzymes synthesis (29). The production of biosurfactants can also act as antagonistic to pathogenic and mycotoxic fungi (121).

In this work, the biocontrol effect was tested against *Alternaria sp..* This mold was chosen because it is a widely distributed pathogen that can cause considerable crop losses under field conditions (108). However, this approach only allowed the testing of isolates under moderate salinity conditions (SA20 medium) since sodium chloride directly inhibits the growth of *Alternaria*. It as has been reported in previous studies indicating that increased salinity has an inhibitory effect on microbial activity (129). The results to the inhibition of growth of the mycelium, expressed as percentage in relation to the growth in the negative control. Sterilized dH_2O was used as negative control and fluconazole commercial disks as a positive control (Figure 3).

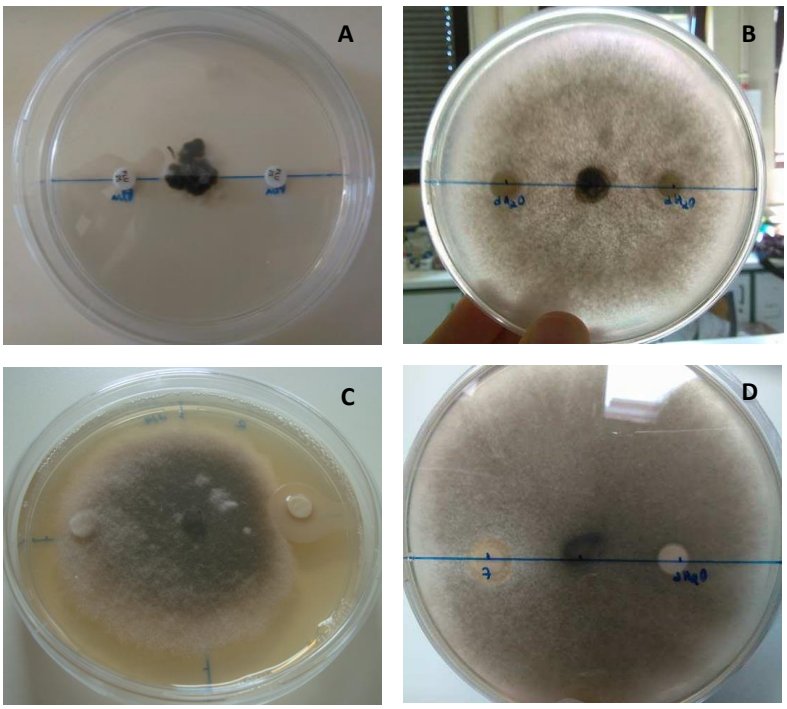

Figure 3. Biocontrol effect against *Alternaria sp..* **A** - Fluconazole (positive control) **B** - Sterilized dH_2O (negative control) **C** - positive result in #2 *Idiomarina sp.* **D** - negative result in #7 *Bacillus licheniformis*

The results are summarized in table 4. As expected, fluconazole caused a strong inhibition of growth (82.2%) and no inhibition was observed in the negative control. Among the tested isolates*, Idiomarina* species caused the strongest biocontrol effect on *Alternaria* (34.4%-59.2% inhibition). The range of percent inhibition caused by *Marinobacter* was 10.7%-38.5%. The production of bioactive substances against the genus *Alternaria* had already been reported in species of *Marinobacter* isolated from the Great Salt Plain of Oklahoma (130). *Halobacillus and Bacillus* caused very little (0.0%-21.7%) or no inhibition at all, respectively. Similar results were obtained with Sgroy et al. (2009) (108) with a *Bacillus licheniformis* isolated from the halophyte *Prosopis strombulifera*. According to the results, *Idiomarina* may be regarding a promising biocontrol agent for saline agriculture or for the crop cultivation of halophytes.

Table 4. Percentage inhibition of mycelial growth of *Alternaria sp*. in the solid medium SA20.

Samples	**SA20**
Negative control	0.0 ± 0.0%
Positive control	82.2 ± 4.4%
1 Bacillus licheniformis	0.0 ± 0.0%
2 Idiomarina sp.	47.6 ± 3.7%
3 Idiomarina seosinensis	45.3 ± 13.1%
4 Halobacillus alkaliphilus	0.0 ± 0.0%
5 Halobacillus locisalis	21.7 ± 7.2%
6 Idiomarina seosinensis	48.1 ± 13.9%
7 Bacillus licheniformis	0.0 ± 0.0%
8 Marinobacter salsuginis	38.5 ± 5.6%
9 Marinobacter salsuginis	10.7 ± 7.5%
10 Idiomarina zobellii	34.4 ± 5.7%
11 Marinobacter adhaerens	38.4 ± 15.4%
12 Idiomarina seosinensis	57.8± 5.6%
13 Idiomarina seosinensis	43.6 ± 11.7%
14 Idiomarina zobellii	59.2 ± 8.7%

Microorganisms produce siderophores as a strategy to optimize iron uptake under iron limitation (131). Bacterial siderophores play an important role in promoting plant growth and in controlling several plant pathogens by outcompeting them for iron (46). In this work, siderophore production was detected through the reaction of ferric siderophore complexes with an indicator dye (chrome azurol S) (132). It is a qualitative assay and the isolates were tested only on SA20 medium because of salt precipitation at higher salinities. The formation of a yellow halo around the colonies, or the color change of the medium from blue to yellow was interpreted as a positive result (Figure 4). The color change observed in the medium results in the diffusion of the siderophore produced by the microorganisms in the CAS medium, and the intensity of this change can be related to the concentration of the siderophores (132).

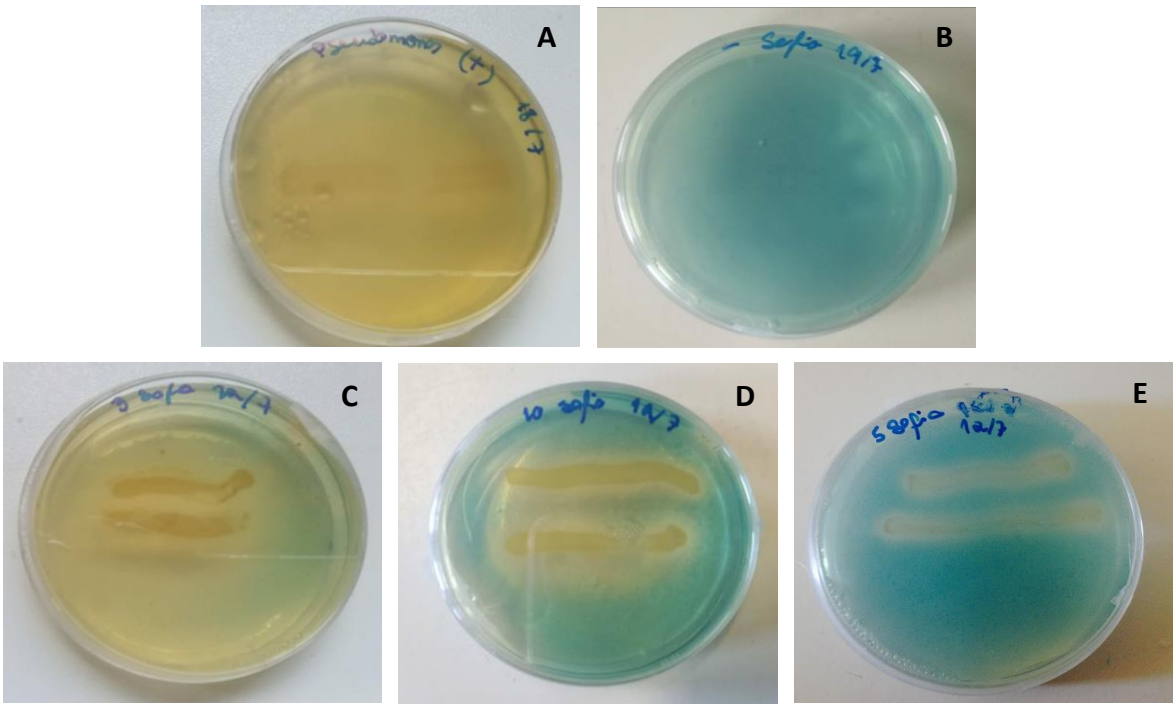

Figure 4. Siderophore production **A -** *Pseudomonas aeruginosa* (positive control) **B -** non-inoculated SA20 (negative control) **C** - positive result (++) in #3 *Idiomarina seosinensis* **D** - positive result (+) in #10 *Marinobacter salsuginis* **E** - positive result (-) in #5 *Halobacillus locisalis*

Non-inoculated SA20 plates were used as negative controls and a siderophore positive strain of *Pseudomonas aeruginosa* was included as positive control (44). The results are summarized in table 5.

Table 5. Siderophore production results in SA20 medium

Samples	Siderophore production
Positive control	++
1 Bacillus licheniformis	+
2 Idiomarina sp.	++
3 Idiomarina seosinensis	++
4 Halobacillus alkaliphilus	-
5 Halobacillus locisalis	-
6 Idiomarina seosinensis	+
7 Bacillus licheniformis	+
8 Marinobacter salsuginis	+
9 Marinobacter salsuginis	+
10 Idiomarina zobellii	+
11 Marinobacter adhaerens	+
12 Idiomarina seosinensis	++
13 Idiomarina seosinensis	+
14 Idiomarina zobellii	+

++ halo formation and color change of the medium
+ formation of a great halo - formation of a small halo

Siderophore production was detected in all isolates. Depending on the chemical nature of the sites of coordination with iron, siderophores can be assigned to into three large groups: catecholates, hydroxamates, and α-carboxylates (108, 131). The production of siderophores in the genus *Halobacillus* was first reported only in 2016 (133), but was previously known in *Idiomarina* (134). The genus *Marinobacter* produces a class of siderophores classified as marinobactins (131), whereas the species *Bacillus licheniformis*

produces a catecholate siderophore called bacillibactin (135). The fact that all isolated expressed siderophores may be related with the adaptation of iron acquisition processes in the extreme osmotic conditions of hypersaline environments (136). As a consequence, the isolates obtained are promising for the mitigation of iron of plants growing in arid or saline soils.

4.4 Quorum quenching

In this work, a qualitative approach used *Chromobacterium violaceum* as a model to test quorum sensing inhibition in Gram negative bacteria. The positive results correspond to the inhibition of the production of violacein (purple pigment) which is regulated by N-hexanoyl-homoserine lactones (C6-HSL) and inhibited by AHL (acylated homoserine lactones) analogues and other antagonists (96) (Figure 5).

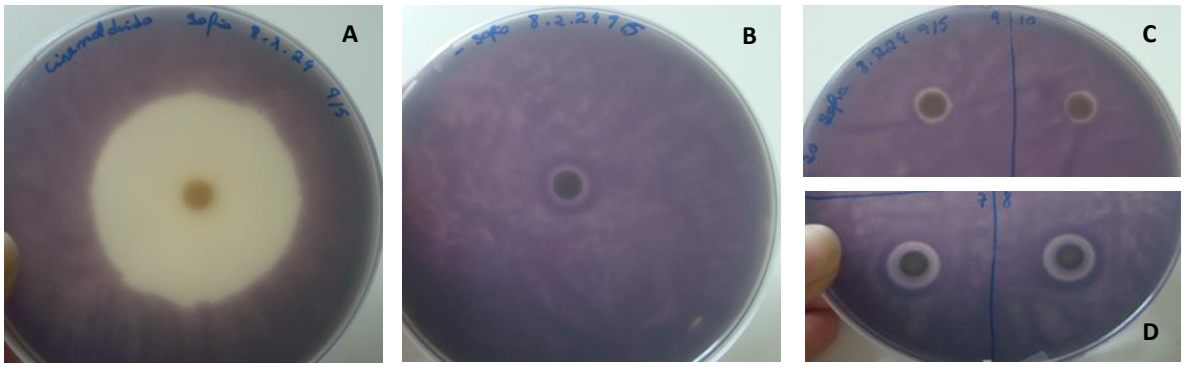

Figure 5. Quorum sensing inhibition in *Chromobacterium violaceum*. **A -** cinnamaldehyde (positive control) **B** - *Chromobacterium violaceum* (negative control) **C** - negative results of #9 *Marinobacter salsuginis* and #10 *Idiomarina zobellii* in SB20 medium **D** - positive results of #7 *Bacillus licheniformis* and #8 *Marinobacter salsuginis* in SB20 medium

The model strain (*C. violaceum*) was used as positive control and cinnamaldehyde, a known inhibitor of QS in Gram negative bacteria (137), was used as positive control. The results obtained are summarized in Table 6.

Quorum sensing inhibition was observed for the two *Bacillus licheniformis* strains, independently of salinity of the medium. *Bacillus licheniformis* is able to inhibit QS through the production of AHL lactonase, an enzyme responsible for AHL degradation

(98). *Idiomarina* strains were variable in terms of quorum quenching and also in terms of the effect of salinity. In some strains, no inhibition was observed (#3 and #14), in others inhibition occurred only at the highest salinity (#10 and #13) and finally others inhibited QS independently of the salinity of the medium (#2, #6 and #12). The results obtained with *Halobacillus* were also variable among strains (positive for #4 and negative for #5) but without a detectable effect of salinity of the cultivation medium. Quorum sensing inhibition by *Halobacillus* is reported in literature (98).

Table 6. Quorum quenching results in SB20 and SB100 media

Samples	**SB20**	**SB100**
Negative control	-	-
Positive control	+	+
1 Bacillus licheniformis	+	+
2 Idiomarina sp.	+	+
3 Idiomarina seosinensis	-	-
4 Halobacillus alkaliphilus	+	+
5 Halobacillus locisalis	-	-
6 Idiomarina seosinensis	+	+
7 Bacillus licheniformis	+	+
8 Marinobacter salsuginis	+	+
9 Marinobacter salsuginis	-	-
10 Idiomarina zobellii	-	+
11 Marinobacter adhaerens	+	-
12 Idiomarina seosinensis	+	+
13 Idiomarina seosinensis	-	+
14 Idiomarina zobellii	-	-

- no inhibition + inhibition

Marinobacter also produced variable results, depending on the salinity of the culture medium. Inhibition of QS occurred in both salinities with strain #8, only in the

lowest salinity in strain #11 or was not observed with strain #9. Diketopiperazines (DKPs), are known as quorum-sensing bacterial sensors and have already been isolated in *Marinobacter* where their QS-inhibitory activities have been demonstrated (137, 138).

Biosurfactants can be considered as quorum sensing antagonists because they interfere with the binding of the auto-inducer to the receptors (98). With the *C. violaceum* bioassay it is not possible to establish the mechanisms of quorum quenching but considering that biosurfactant production was not detected in any of the tested strains, it is unlikely that the observed QS inhibition was mediated by biosurfactants but rather by other types of molecules.

4.5 Activity of extracellular hydrolytic enzymes

The maximum rate of hydrolysis was calculated using the concentrations determined in the saturation assay, which were 0.27 mM for MUF-phosphate and 0.05 mM for MUF-stearate. The results are summarized in table 7. The maximum potential hydrolysis rate was 0.0231 nmol/h*CFU for esterase (*Halobacillus alkaliphilus*) and 0.0495 nmol/h*CFU for phosphatase (*Halobacillus alkaliphilus*)

Genus *Halobacillus* presented the highest overall hydrolytic potential. On average, esterase activity of *Halobacillus* strains (0.0213 nmol/h*CFU) was higher than for other genera (ANOVA, $p < 0.05$). Phosphatase activity of *Halobacillus* strains (0.0336 nmol/h*CFU) higher than that of *Bacillus* and *Idiomarina* (ANOVA $p < 0.05$), but not significantly different from *Marinobacter* (ANOVA, $p > 0.05$).

Phosphatase activity allows bacteria to obtain phosphate from organic sources alleviating competition with plants for inorganic phosphate, under phosphorus-limiting conditions (23). The production of esterases may also function as a biocontrol mechanism, since these enzymes are able to mediate some of the mechanisms associated with antagonistic relations involving phytopathogenic fungi (29). Esterase activity is involved in the detoxification of albicidin, a phytotoxin produced by several plant pathogens like *Xanthomonas albilineans* that attacks sugar-cane (140). Thus, the isolates with the highest hydrolytic potential may be more promising as biocontrol agents.

Table 7. Maximum hydrolysis rate of extracellular phosphatases (hydrolysis of MUF-phosphate) and esterases (hydrolysis of MUF-stearate) standardized by the concentration of viable cells (CFU).

Samples	MUF-phosphate (nmol/h*CFU)	MUF-stearate (nmol/h*CFU)
1 Bacillus licheniformis	0.0107±0.0016	0.0081±0.0020
2 Idiomarina sp.	0.0044±0.0018	0.0038±0.0002
3 Idiomarina seosinensis	0.0113±0.0039	0.0052±0.0005
4 Halobacillus alkaliphilus	0.0495±0.0074	0.0231±0.0021
5 Halobacillus locisalis	0.0177±0.0104	0.0194±0.0021
6 Idiomarina seosinensis	0.0206±0.0020	0.0029±0.0001
7 Bacillus licheniformis	0.0098±0.0028	0.0115±0.0008
8 Marinobacter salsuginis	0.0119±0.0029	0.0076 ±0.0004
9 Marinobacter salsuginis	0.0135±0.0025	0.0117±0.0005
10 Idiomarina zobellii	0.0074±0.0032	0.0116±0.0005
11 Marinobacter adhaerens	0.0173±0.0013	0.0121±0.0003
12 Idiomarina seosinensis	0.0227±0.0057	0.0056±0.0001
13 Idiomarina seosinensis	0.0156±0.0039	0.0125±0.0003
14 Idiomarina zobellii	0.0130±0.0056	0.0089±0.0003

Conclusion

5. Conclusion

In this work, the isolation halophilic bacteria from salt pans of Ria de Aveiro was successfully achieved. A set of 14 strains was assigned to genera *Bacillus*, *Halobacillus*, *Marinobacter* and *Idiomarina*, already reported in the literature as including halophilic species. The halophilic character of the isolates was confirmed by their strict requirement for salt in the cultivation media. This is the first work in which halophilic bacteria were successfully isolated and identified in one of the few active salt pans of the Ria de Aveiro.

Biosurfactants that maintain stability in extreme industrial conditions have been intensively investigated in different groups of extremophile microorganisms and also represented one of the objectives of this work. Although none of the isolates provided evidence of biosurfactant production, they displayed a set of other interesting physiological and biochemical traits, namely in terms of iron acquisition systems and mechanisms of biological antagonism that make them worth testing for plant-growth promotion applications.

A culture-dependent approach was used because this work aimed at exploring the biotechnological potential of these microorganisms. However, this approach had the limitation of targeting a small fraction of the whole bacterial community. In order to gain a more complete picture of the microbial diversity present in the hypersaline microbial habitat of the active salt pans, a culture-independent approach, namely involving advanced high throughput sequencing techniques would be necessary. This could also provide information on particular prokaryote groups, namely Archaea that are expected to be well represented in such an extreme environment but were not retrieved with the adopted isolation strategy.

As a continuation of the present work, the strains are being characterized in terms of a wider array of extracellular enzymatic activities, production of phytohormones and stress mitigation effect and will be tested for their growth promoting effect on crop cultivation of halophytes.

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7. Appendix A

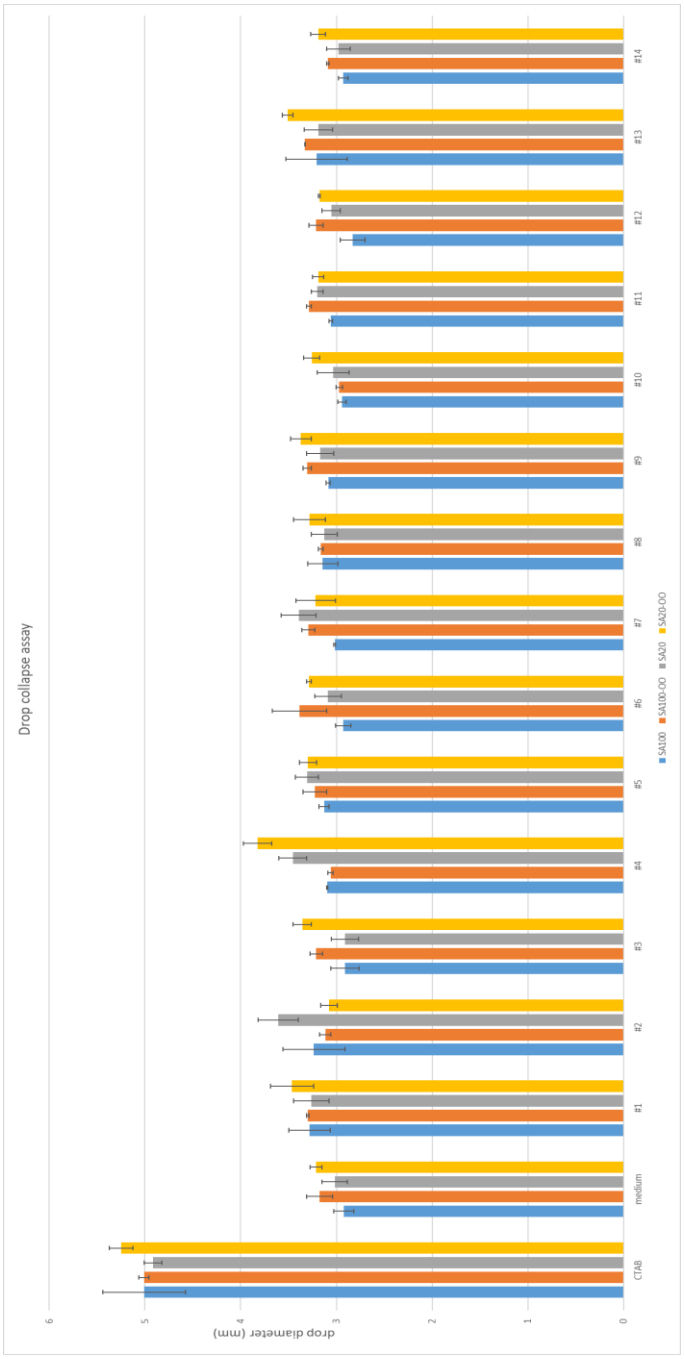

Figure 1. Results of the drop collapse assay expressed as the diameter of the water droplets. Tests correspond to cultures in media SA 100, SA 100-OO, SA 20, SA 20-OO. CTAB (0.2 mM) was used as positive control and non-inoculated culture medium was used as negative control. The bars represent the standard deviation. The values are presented in mm.

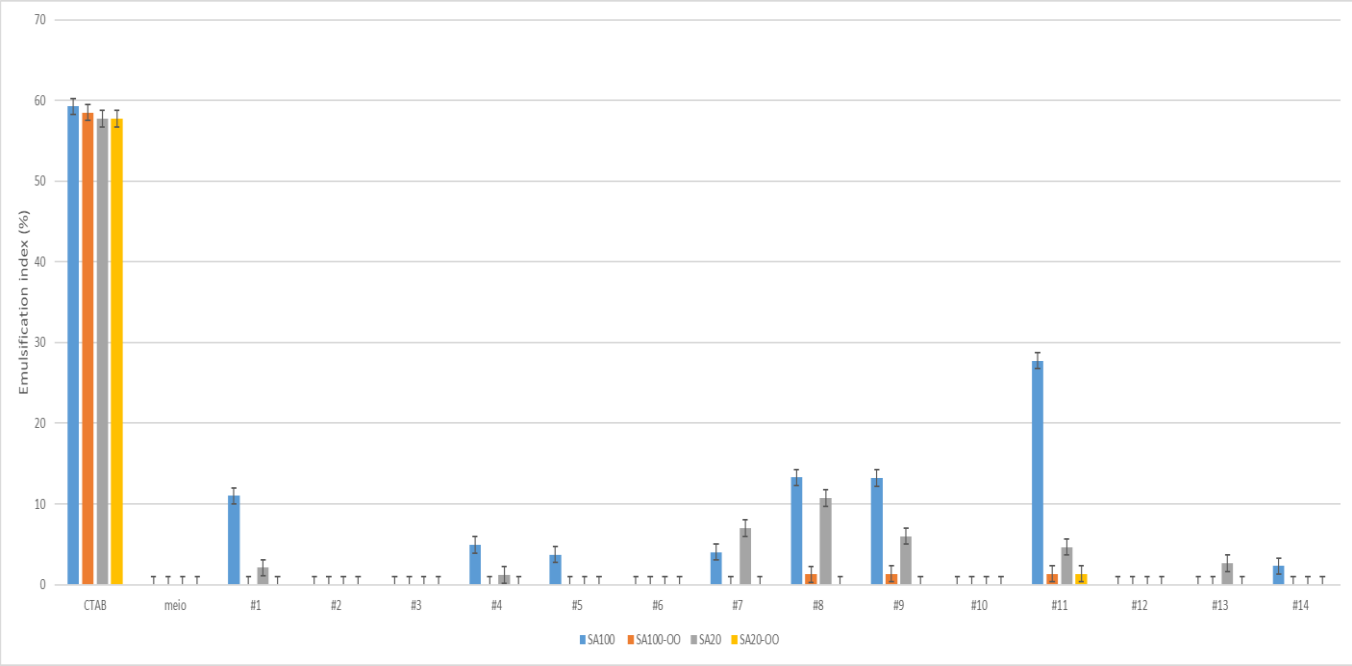

Figure 2. Results of the Emulsification index. Tests correspond to cultures in media SA 100, SA 100-OO, SA 20, SA 20-OO. CTAB (0.2 mM) was used as positive control and medium was used as negative control. The bars represent the standard deviation. The values are presented in percentage.